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Specificity of cGMP binding to a purified cGMP-stimulated phosphodiesterase from bovine adrenal tissue

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The binding of [³H]cGMP (guanosine 3',5'-monophosphate) to purified bovine adrenal cGMP-stimulated phosphodiesterase was measured by Millipore filtration on cellulose ester filter. [³H]cGMP-binding activity was enhanced when the assay was terminated in buffer containing 70% of saturated ammonium sulfate to dilute the enzyme and wash the filters. The cGMP-binding activity was co-purified with the phosphodiesterase activity. The binding of [³H]cGMP to purified enzyme was measured in the presence or absence of the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine. 1-Methyl-3-isobutylxanthine showed linear competitive inhibition with respect to cGMP as substrate in the phosphodiesterase reaction but stimulated the [³H]cGMP-binding activity in the binding assay. The stimulatory effect appeared not to be the result of preservation from [³H]cGMP hydrolysis; no cGMP phosphodiesterase activity has been measured under the cGMP-binding assay conditions, in the absence or presence of the inhibitor. Half-maximal stimulation by 1-methyl-3-isobutylxanthine occurred in the 5–7 μ M concentration range. The specificity of binding of [³H]cGMP was investigated by adding increasing concentration of unlabeled analogs of cAMP (adenosine 3',5'-monophosphate) and cGMP. The binding of [³H]cGMP (50 nM) was displaced by unlabeled cGMP and cAMP with the following potency: 50% displacement was reached at the 0.1 μ M cGMP range and only at a fiftyfold higher cAMP concentration. Our data with comparative series of analogs (e.g. 5'-amino-5'-deoxyguanosine 3',5'-monophosphate and 3'-amino-3'-deoxyguanosine 3',5'-monophosphate) showed that the potencies of stimulation of cAMP phosphodiesterase activity parallels displacement curves or [³H]cGMP binding to purified enzyme with no correlation with phosphodiesterase inhibition sequences. Those experiments suggest that the cGMP-binding activity is directly related to the non-catalytic (allosteric) cGMP-binding site.

Cyclic nucleotide phosphodiesterases exist in multiple forms that can be distinguished by multiple criteria such as substrate specificity, chromatographic, immunological or kinetic properties and control mechanisms. Phosphodiesterase enzymes have been tentatively classified as Ca²⁺/calmodulin-sensitive, cGMP-binding, cAMP-specific and non-specific cyclic nucleotide phosphodiesterases (for review, see [1]). At present, three isozymes of phosphodiesterases appear to bind cGMP with high affinity: one type in rat platelets or lung [2–4], another type in rod outer segments [5, 6] and finally the cGMP-stimulated phosphodiesterase present in many mammalian tissues [7–10]. However, among these enzymes only the phosphodiesterase in rod outer segments and the cGMP-stimulated phosphodiesterase have been

purified to apparent homogeneity [5, 11, 12]. Immunological studies have shown that these two phosphodiesterases are different proteins [13]. The cGMP-binding phosphodiesterase activity in rat platelets or rat lung and in rod outer segments share the following properties: (i) phosphodiesterase activity is co-purified with cGMP-binding activity; (ii) phosphodiesterase activity is separated from protein kinase activity and (iii) cGMP-binding activity is stimulated *in vitro* by phosphodiesterase inhibitors, e.g. 1-methyl-3-isobutylxanthine [2–5].

We have reported previously that: (a) cyclic nucleotide analogs can be useful probes of catalytic and regulatory (or allosteric) sites on the cGMP-stimulated phosphodiesterase, i.e. analogs are found that are potent activators but do not bind to catalytic binding sites and *vice versa* [14, 15], and (b) kinetics in the presence of cGMP or 1-methyl-3-isobutylxanthine (IBMX) also argue in favour of a distinction between allosteric and catalytic binding sites [16]. These data were obtained with a partially purified cGMP-stimulated phosphodiesterase from rat liver.

The cGMP-stimulated phosphodiesterase has been purified to apparent homogeneity from respectively bovine adrenal tissue [11] and calf liver [12]. In bovine adrenal, the purified enzyme has been reported to bind cGMP assessed by Millipore filtration [11]. cGMP-binding activity co-migrated with phosphodiesterase activity under different experimental conditions (e.g. polyacrylamide gradient gel electrophoresis under non-denaturing conditions [11]). In the binding of cGMP to the cGMP-stimulated phosphodiesterase, it has not

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Abbreviations. cAMP, adenosine 3',5'-monophosphate; cGMP, guanosine 3',5'-monophosphate; 5'GMP, guanosine 5'-monophosphate; 3'NH-cAMP, 3'-amino-3'-deoxyadenosine 3',5'-monophosphate; 3'NH-cGMP, 3'-amino-3'-deoxyguanosine 3',5'-monophosphate; 5'NH-cAMP, 5'-amino-5'-deoxyadenosine 3',5'-monophosphate; 5'NH-cGMP, 5'-amino-5'-deoxyguanosine 3',5'-monophosphate; IBMX, 1-methyl-3-isobutylxanthine; QAE-Sephadex, quaternary diethyl-(2-hydroxypropyl)aminoethyl-Sephadex; PhMeSO₂F, phenylmethylsulfonyl fluoride.

Enzymes. Cyclic nucleotide phosphodiesterase or 3',5'-cyclic nucleotide 5'-nucleotidohydrolase (EC 3.1.4.17); 5'-nucleotidase or 5'-ribonucleotide phosphohydrolase (EC 3.1.3.5).

been established which type of binding site (catalytic or allosteric) is involved not has the influence (if any) of IBMX been reported.

These questions were addressed in the present study with the purified adrenal cGMP-stimulated phosphodiesterase. The cyclic nucleotide analogs previously used to map catalytic and non-catalytic binding sites [16] were taken to characterize the specificity of the cGMP-binding activity. Our data show that the potencies of stimulation of cAMP phosphodiesterase activity by cyclic nucleotides analogs parallels displacement curves of [3 H]cGMP binding to purified enzyme with no correlation with phosphodiesterase inhibition sequences.

MATERIALS AND METHODS

Purification of the bovine adrenal cGMP-stimulated phosphodiesterase

The cGMP-stimulated phosphodiesterase was purified from bovine adrenal tissue follows: all operations were carried out at 4°C; 300 g whole frozen bovine adrenal tissue were homogenized in a teflon-glass homogenizer in 1.2 l buffer containing 20 mM Tris/HCl pH 7.5, 2 mM MgCl₂, 0.4 mM PhMeSO₂F, 2 mM benzamine, 5 µM leupeptin, 25 mg/l trypsin inhibitor, 5 mM 2-mercaptoethanol (buffer A) and 0.25 M sucrose. The homogenate was centrifuged at 33000 × g for 1 h and the supernatant was decanted through two layers of cheese cloth. 1 l of the supernatant was loaded on to a DEAE-cellulose (DE-52) column (30 × 5 cm) equilibrated in buffer A. Phosphodiesterase activity was eluted by an exponential gradient of 0–0.5 M ammonium sulfate in buffer A. The fractions with cGMP-stimulated phosphodiesterase activity were immediately concentrated by ammonium sulfate precipitation at 60% saturation and dialyzed overnight as previously described [14]. The enzyme was made 2 mM dithiothreitol, 1 mM EDTA and 15 mM benzamidine prior to its application to the cGMP epoxy-activated Sepharose 6B column (12 × 1 cm) prepared as in [11]. The affinity column was first washed with 20 ml 20 mM Tris/HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol (buffer B) and with 10 ml of buffer B containing 125 mM NaCl. The washed resin was warmed to 23°C and rinsed with buffer B containing 125 mM NaCl and 10 µM cAMP. The bulk of phosphodiesterase activity was further eluted with the same buffer including 10 mM cAMP. cAMP was removed from the phosphodiesterase by dialysis in a Micro ultrafiltration Amicon cell (8 MC). Specific activity of the affinity purified enzyme ranged from 60–100 µmol cGMP hydrolyzed × min⁻¹ × mg protein⁻¹ with 40 µM cGMP as substrate. The preparation was in a state approaching homogeneity as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and its mobility corresponded to an apparent of M_r 105000 (Fig. 1).

cGMP-binding and phosphodiesterase activities comigrated after gel filtration on a Sephacryl S-200 column (90 × 0.9 cm) (data not shown). Since the cGMP-binding assay was performed in the presence of bovine serum albumin, the enzyme was stored at –80°C in the presence of bovine serum albumin at 2 mg/ml.

DEAE-cellulose chromatography of purified cGMP-stimulated phosphodiesterase from bovine adrenal tissue

Purified cGMP-stimulated phosphodiesterase (150 µg) was applied to a DEAE-cellulose DE-52 column (5 × 0.4 cm).

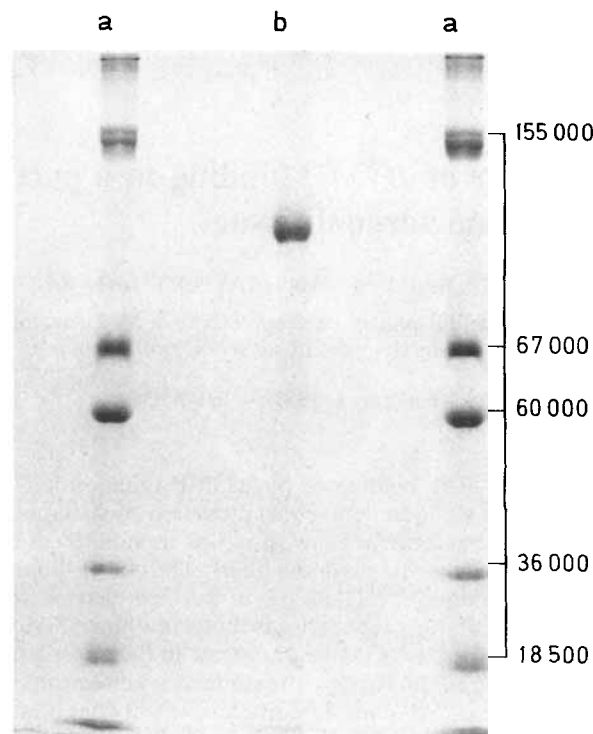


Fig. 1. Sodium dodecyl sulfate/gel electrophoresis of the purified bovine adrenal cGMP-stimulated phosphodiesterase. About 7 µg of protein were applied to the 10% polyacrylamide gel (line b). The relative molecular mass based on the migration of polymerase β , bovine serum albumin, catalase, lactate dehydrogenase, ferritin (5 µg of each, line a) was approximately 105000. The gels were stained with Coomassie blue R-250 and destained by diffusion first in 20% methanol/10% acetic acid and subsequently in 50% methanol/10% acetic acid/0.5% glycerol solutions

The column was washed with 2 vol. of 20 mM Tris/HCl pH 7.5 buffer containing 2 mM MgCl₂, 0.4 mM PhMeSO₂F, 15 mM benzamidine, 5 µM leupeptin, 25 mg/l soybean trypsin inhibitor, 5 mM 2-mercaptoethanol. Phosphodiesterase and [3 H]cGMP-binding activities were co-eluted with a linear gradient of NaCl 125–500 mM (9 ml:9 ml).

Phosphodiesterase assay

Unless otherwise specified, phosphodiesterase activity was assayed as described in [9].

cGMP-binding assay

cGMP binding was determined by Millipore filtration on cellulose ester filter (0.45 µm). Purified phosphodiesterase was incubated in 20 mM Tris/HCl pH 7.5, 200 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin and [3 H]cGMP in a final volume of 0.1 ml. Unless otherwise specified, incubation was performed at 0°C for 10 min. Each sample was diluted with 1 ml of an ice-cold buffer medium containing 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 200 mM NaCl, 1 mM dithiothreitol (buffer C) and 70% of saturated ammonium sulfate and the mixture filtered on cellulose ester filters. Filters were then washed twice with 4 ml of the same buffer and counted by liquid scintillation in 6 ml of Insta-Gel. Phosphodiesterase activity under the cGMP-binding assay conditions was not detectable: purified phosphodiesterase

was incubated in the binding assay mixture; after 10 min at 0°C, the pH of the medium was decreased to pH 3 with 40 mM phosphoric acid to block any hydrolytic activity. In control experiments, with the purified cGMP-stimulated phosphodiesterase incubated in a standard phosphodiesterase mixture at 30°C [9], we have checked that lowering the pH did indeed block phosphodiesterase activity. After addition of phosphoric acid to pH 3, the separation of cGMP from its eventual reactions product 5'GMP was achieved by two independent procedures: (a) the samples were applied to a reversed phase column (Bondapak C₁₈/porasil B) of 0.3 ml from which [³H]5'GMP was eluted with 1 ml 1% methanol in 10 mM KH₂PO₄/H₃PO₄ buffer at pH 3 and subsequently [³H]cGMP eluted with 1 ml 50% methanol in the same buffer [17]; or (b) the samples were neutralized at pH 7.5 with NaOH, incubated with an excess of 5'-nucleotidase at 30°C for 20 min and then chromatographed on QAE-Sephadex A-25 as in [9]. By these two methods, under binding assay conditions, no phosphodiesterase activity (less than 0.5% cGMP hydrolysis) was measured with the purified cGMP-stimulated phosphodiesterase in the presence or in the absence of IBMX. The identity of bound cGMP to the cGMP-stimulated phosphodiesterase was checked by high-performance liquid chromatography on a LiChrosorb 10 RP 18 column; the mobile phase was 10 mM KH₂PO₄/Na₂HPO₄ in 10% methanol at pH 6.5 [18].

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate gel electrophoresis was performed by the method of Laemmli [19] using a 10% resolving gel and a 5% stacking gel 1.5-mm thick. Electrophoresis was carried out at 4°C for 6 h at 150 V.

Protein determination

Protein determinations were carried out according to the method of Lowry [20] using bovine serum albumin (99% pure from Serva) as standard.

Materials

[8-³H]cGMP (specific activity 15 Ci/mmol) was obtained from Amersham International and purified on a 0.5 ml reversed-phase column (Bondapak C₁₈/porasil B) equilibrated with 3 ml 20 mM Tris/HCl pH 7, 1% methanol. 15 µl [8-³H]cGMP diluted to 200 µl with 20 mM Tris/HCl pH 7 were applied to the column. The column was washed with 1 ml 20 mM Tris/HCl pH 7, 1% methanol. [³H]cGMP was eluted with 2 ml 20 mM Tris/HCl pH 5, 5% methanol and the radioactive fractions were pooled [17]. cAMP, cGMP, 5'GMP and guanosine were purchased from Boehringer (Mannheim). DEAE-cellulose (DE-52) was from Whatman. Cellulose ester filters (HAWP 29324) were from Millipore. 5'-Nucleotidase (*Crotalus atrox* venom), PhMeSO₂F, IBMX, leupeptin and dithiothreitol were purchased from Sigma Chemical Company (St Louis). Bondapak C₁₈/Porasil B, 35–75 µm, was obtained from Waters Associates Inc. (Milford). QAE-Sephadex A-25, epoxy-activated Sepharose 6B and relative molecular mass standards for electrophoresis were from Pharmacia (Uppsala). Insta-Gel was from Packard. Fatty-acid-poor bovine albumin (99% pure) was purchased from Serva (Heidelberg). 3'NH-cAMP, 3'NH-cGMP, 5'NH-cAMP, 5'NH-cGMP, purine ribonucleoside 3',5'-monophosphate and benzimidazole ribonucleoside 3',5'-mono-

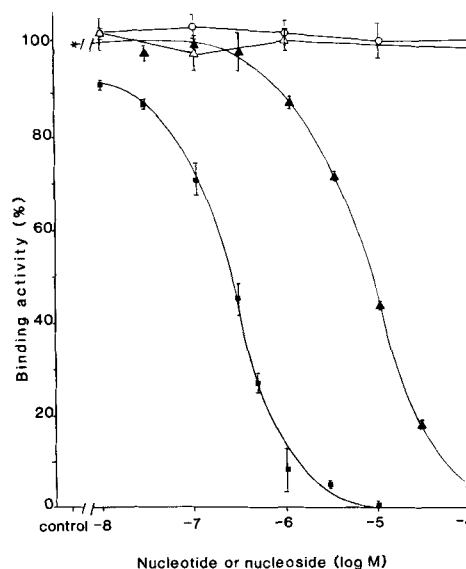


Fig. 2. Displacement of [³H]cGMP binding to purified GMP-stimulated phosphodiesterase by unlabeled compounds. The enzyme was incubated for 10 min at 0°C in the presence of 50 nM [³H]cGMP and increasing concentrations of unlabeled cGMP (■), cAMP (▲), 5'GMP (△) and guanosine (○). The binding assay was conducted as outlined in Materials and Methods in the presence of 1 mM IBMX. Results are the means of triplicates and are expressed as a percentage of control activity ± SEM (the control activity is [³H]cGMP bound in the absence of unlabeled cyclic nucleotide). The data are representative of experiments with three different preparations of purified enzyme.

phosphate were synthesized as published previously [21–24] and kindly provided by Dr B. Jastorff (University of Bremen).

RESULTS

General characterization of [³H]cGMP binding to the purified cGMP-stimulated phosphodiesterase

The binding of [³H]cGMP to the purified phosphodiesterase was examined by Millipore filtration as described in Materials and Methods. The following experiments were done in the presence of 1 mM IBMX in the incubation medium. Under these assay conditions: the binding reaction of [³H]cGMP was time-dependent and reached a plateau after 5–10 min. By the mere addition of 70% of saturated ammonium sulfate in the stop and wash buffer (i.e. buffer C: 20 mM Tris/HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol), the [³H]cGMP-binding activity was increased by 150% (not shown). We therefore systematically used those conditions in all our binding assays. The binding of 50 nM [³H]cGMP to the purified phosphodiesterase was displaced by unlabeled cGMP and cAMP with the following potency: 50% displacement was reached at 0.1 µM cGMP but only at fiftyfold higher cAMP concentration. In contrast, [³H]cGMP binding was not displaced by 5'GMP or guanosine in the 0.01–100 µM range (Fig. 2).

Stimulation by IBMX of [³H]cGMP-binding activity to the purified cGMP-stimulated phosphodiesterase

The purified bovine adrenal cGMP-stimulated phosphodiesterase exhibited properties similar to those reported previously on partially purified enzymes [14–16, 25]. At 3 µM

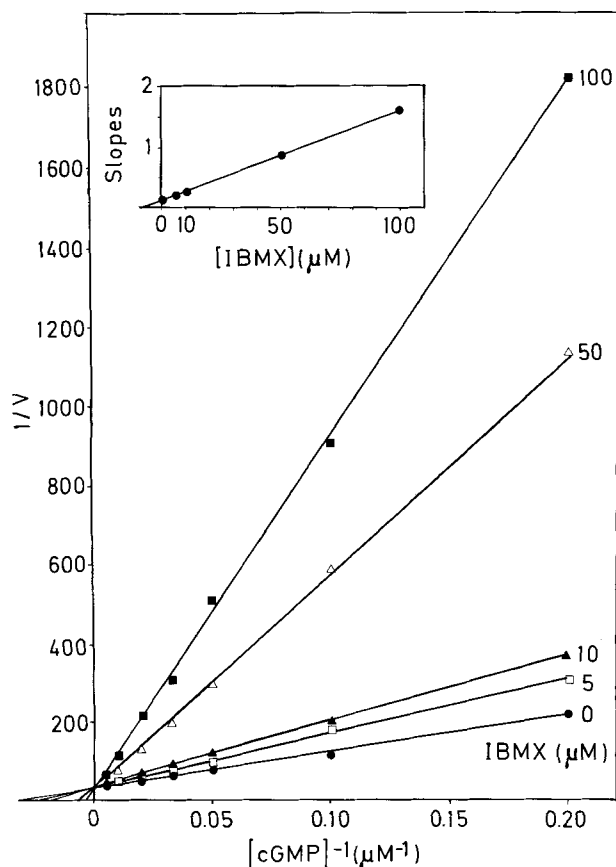


Fig. 3. Inhibitory effect of IBMX on cGMP hydrolysis by the adrenal cGMP-stimulated phosphodiesterase (double-reciprocal plot). Activity was measured in the 5–200 μM range for cGMP. The inset shows the replot of the slopes of the primary plot as a function of inhibitor concentrations. v is expressed as $\text{mmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$

cAMP, cAMP phosphodiesterase activity was stimulated by increasing the concentration of cGMP: a twofold stimulation of cAMP phosphodiesterase activity occurred generally in the 0.1 μM cGMP range (Table 1). The enzyme displayed positive homotropic cooperativity with a Hill coefficient of 1.9 for cAMP hydrolysis (not shown). In contrast, on a fully activated enzyme, with cAMP as variable substrate and in the presence of 3 μM cGMP, double-reciprocal plots of cGMP-stimulated phosphodiesterase were linear. Michaelian kinetics were also observed with cGMP as substrate in the 5–200 μM range. Inhibition by IBMX was shown to be competitive with respect to cGMP. A replot of the slopes of the primary reciprocal plot was linear and a K_i value of 8 μM for IBMX was calculated (Fig. 3, inset).

The influence of IBMX on cGMP-binding activity was studied with the purified phosphodiesterase. In the binding assay, no hydrolysis of cGMP was detected in the presence or absence of IBMX (see Materials and Methods). [^3H]cGMP-binding activity was stimulated in the presence of 1 mM IBMX in the incubation medium. Stimulation of cGMP binding at 50 nM [^3H]cGMP appeared at a concentration as low as 3 μM IBMX and was maximal at 100 μM . Half-maximum stimulation occurred in the 5–7 μM range with three different phosphodiesterase preparations (Fig. 4, inset). When purified cGMP-stimulated phosphodiesterase was applied to a DEAE-cellulose column, the phosphodiesterase activity as well as the basal and IBMX-stimulated cGMP-

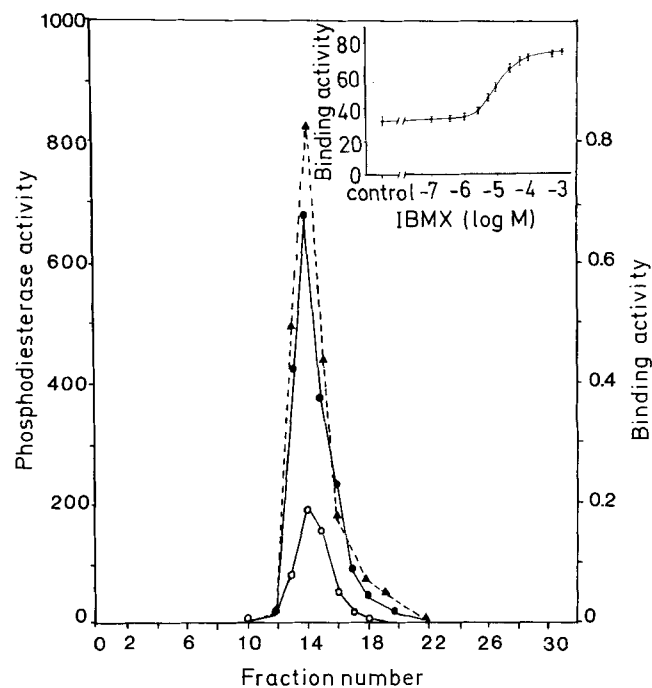


Fig. 4. DEAE-cellulose chromatography of purified cGMP-stimulated phosphodiesterase from bovine adrenal tissue. Enzyme (150 μg) was applied to DEAE-cellulose column (0.4 \times 5 cm) and was eluted by a linear gradient of NaCl 125–500 mM (9 ml:9 ml) starting at fraction 11. Fractions of 300 μl were collected. Phosphodiesterase activity (\blacktriangle — \blacktriangle) and cGMP-binding activities in the absence (\circ — \circ) or in the presence (\bullet — \bullet) of 1 mM IBMX were measured as described in Materials and Methods. Phosphodiesterase activity was assessed at 40 μM cGMP as substrate and expressed as pmol cGMP hydrolyzed $\times 5 \mu\text{l}^{-1} \times 5 \text{min}^{-1}$. Binding activity was measured at 50 nM [^3H]cGMP in the absence or in the presence of 1 mM IBMX and expressed as pmol cGMP bound $\times 80 \mu\text{l}^{-1} \times 10 \text{min}^{-1}$. The inset shows the dose-response curve between 50 nM [^3H]cGMP-binding activity and the concentration of IBMX. Results (triplicates) are expressed as pmol cGMP bound/mg protein \pm SEM

binding activities co-migrated by elution with a linear salt gradient (Fig. 4).

The potencies of stimulation of cAMP phosphodiesterase activity parallels displacement curves of [^3H]cGMP binding to purified cGMP-stimulated phosphodiesterase

As with the crude rat liver cGMP-stimulated phosphodiesterase [14], the purified enzyme from bovine adrenal was stimulated by cyclic nucleotide derivatives in a concentration-dependent manner. These data were obtained in the absence of cGMP and at 3 μM cAMP substrate level (Fig. 5A and Table 1). We previously compared the inhibition pattern of phosphodiesterase activity to the activating potencies of cyclic nucleotide analogs. In particular, 3'-amido and 5'-amido derivatives of cAMP and the two base-modified analogs, benzimidazole ribonucleoside 3',5'-monophosphate and purine nucleoside 3',5'-monophosphate, were used as a tool to distinguish between catalytic and non-catalytic binding sites [14, 15]. In the present study, we have compared activation and inhibition of phosphodiesterase activity to binding studies in which analogs were tested as competitors of labeled cGMP binding. Those binding experiments were performed at 30–60 nM [^3H]cGMP with increasing concentrations of unlabeled analog in the 0.01–100 μM range. A typical exper-

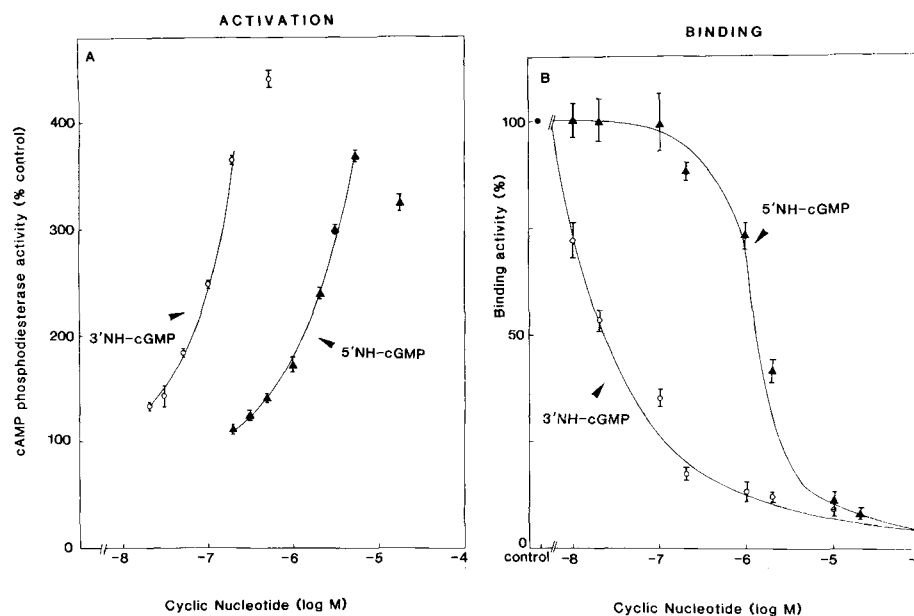


Fig. 5. Dose-response between the activation of cAMP phosphodiesterase activity at 3 μ M and the concentration of unlabeled 3'NH-cGMP and 5'NH-cGMP (A) and displacement of 40 nM [3 H]cGMP binding to purified enzyme by 3'NH-cGMP and 5'NH-cGMP (B). (A) Results (triplicates) are expressed as a percentage of control values \pm SEM (control measured without analog). (B) The binding assay is conducted in the presence of 1 mM IBMX. Results (triplicates) are expressed as in Fig. 4 \pm SEM

iment is illustrated in Fig. 5B with 3'NH-cGMP and 5'NH-cGMP. Fig. 5A compares the same analogs tested as activators of cAMP phosphodiesterase activity. 3'NH-cGMP was less potent than 5'NH-cGMP as an inhibitor of phosphodiesterase activity (Table 1). This was in contrast to the binding data where 3'NH-cGMP did preferentially displace cGMP binding relative to 5'NH-cGMP. 50% displacement was obtained at 0.03 μ M and 1.5 μ M for 3'NH-cGMP and 5'NH-cGMP, respectively (Table 1, Fig. 5B). 3'NH-cGMP was also more potent, relative to 5'NH-cGMP, when checked as an activator (Table 1, Fig. 5A).

A similar conclusion was reached from the comparison between 5'NH-cAMP and 3'NH-cAMP (Table 1). cBIMP was also more potent than cPMP as an activator of phosphodiesterase activity, but in contrast the inhibition pattern of phosphodiesterase activity was reversed. However, the [3 H]cGMP-binding activity was displaced by the same sequence as the stimulation of enzyme activity (Table 1).

DISCUSSION

The purification of bovine adrenal cGMP-stimulated phosphodiesterase is achieved by DEAE-cellulose chromatography, ammonium sulfate precipitation and affinity chromatography on cGMP-Sepharose. Elution of the column is performed in two steps: with 10 μ M cAMP, no phosphodiesterase activity is released from the column but a protein-staining band at a relative molecular mass of approximately 48000 (not shown). The bulk of phosphodiesterase activity is further and identically eluted with 10 mM of either cGMP or cAMP. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis reveals a single protein-staining band with a relative molecular mass of approximately 105000 in agreement with the previous report of Martins et al. [11]. Phosphodiesterase and cGMP-binding activities comigrate after DEAE-cellulose chromatography, suggesting that both activities reside in the same molecule.

Table 1. Binding, activation and inhibition properties of cyclic nucleotide analogs

Analogues tested in the 0.01–100 μ M range. The data are representative of experiments with three different preparations of purified enzyme and are expressed as means \pm SEM. Binding is expressed as the concentration necessary to reach 50% displacement of 30–60 nM [3 H]cGMP binding. Activation is expressed as the concentration necessary to reach a twofold stimulation of phosphodiesterase activity at 3 μ M cAMP; n.d., no detectable activation with 5'NH-cAMP. For inhibition, cGMP phosphodiesterase activity was measured with 10 μ M cGMP substrate level in the presence or absence of analog. Results are expressed as a percentage of control (cGMP enzymatic hydrolysis without any addition as in [14]). cBIMP, benzimidazole ribonucleoside 3',5'-monophosphate; cPMP, purine ribonucleoside 3',5'-monophosphate

Analog	Binding	Activation	Inhibition
	μ M	μ M	% control
cGMP	0.09 ± 0.01	0.09 ± 0.02	—
3'NH-cGMP	0.03 ± 0.01	0.06 ± 0.01	86 ± 1
3'NH-cAMP	1.5 ± 0.5	3.5 ± 0.5	70 ± 2
5'NH-cGMP	1.5 ± 0.5	1.5 ± 0.5	15 ± 1
5'NH-cAMP	> 100	n. d.	11 ± 0.5
cBIMP	1.25 ± 0.30	1.25 ± 0.25	88 ± 1
cPMP	7.00 ± 2.02	9.5 ± 0.5	72 ± 1

The binding of [3 H]cGMP to the cGMP-stimulated phosphodiesterase measured by Millipore filtration is largely dependent on the experimental conditions. In particular, it is increased by the mere addition of 70% of saturated ammonium sulfate in the stop and wash buffer after the incubation is terminated. The same procedure has been previously used in a cGMP assay to increase the binding of cGMP to the lobster cGMP-dependent protein kinase ([26] and Decoster, personal communication). Likewise, studies with bovine lung cGMP-dependent protein kinase have shown that cGMP bound to one of the two types of binding sites dissociates

rapidly and that the inclusion of ammonium sulfate in the wash buffer prevents this phenomenon [27, 28].

The properties of the purified adrenal phosphodiesterase appear similar if not identical to those of partially purified cGMP-stimulated phosphodiesterase in rat liver supernatant and particulate fractions [14, 15, 29]. cGMP and its analogs stimulate cAMP hydrolysis and kinetics with respect to cAMP or cGMP as substrate show positive homotropic cooperativity. With the partially purified rat liver enzyme, we reported that inhibition and activation potencies of multiple analogs of cyclic nucleotides were not correlated [14–16]. These data argued in favour of a distinction between catalytic and non-catalytic (allosteric) binding sites. In this study, we have addressed the question of whether the cGMP-binding activity, measured by Millipore filtration, results from an interaction of cGMP to the allosteric site or to the catalytic site. As shown here, the cGMP-binding activity is enhanced in the presence of the phosphodiesterase inhibitor, IBMX. The mechanism of stimulation is unknown. It does not result from preservation of cGMP hydrolysis by IBMX, because no hydrolysis of cGMP is being measured under our binding assay conditions. IBMX shows linear competitive inhibition with respect to the substrate of the phosphodiesterase reaction. Moreover, increased binding activity in the presence of IBMX is not reversed by increasing the concentration of the phosphodiesterase inhibitor (up to 1 mM IBMX). Thus, it is suggested that the binding of cGMP results from its interaction at non-catalytic binding sites. This is further confirmed by means of cyclic nucleotide analogs. The cGMP-binding activity is cGMP-specific relative to cAMP. Moreover the use of cyclic nucleotide analogs shows that cGMP binding is displaced with a sequence that is not comparable with phosphodiesterase inhibition sequence. For example, 3'NH-cGMP is less potent than 5'NH-cGMP as an inhibitor of phosphodiesterase activity. This is in contrast to the binding data which shows that 3'NH-cGMP does preferentially displace cGMP binding relative to 5'NH-cGMP. 3'NH-cGMP is also more potent as an activator of phosphodiesterase activity. A similar conclusion is reached from the comparison between benzimidazole ribonucleoside 3',5'-monophosphate and purine ribonucleoside 3',5'-monophosphate. Our data with comparative series of analogs show that the potencies of stimulation of cAMP phosphodiesterase activity parallels displacement curves of [³H]cGMP binding to purified cGMP-stimulated phosphodiesterase. Those experiments suggest that the cGMP-binding activity is measured on non-catalytic (allosteric) binding sites.

Stimulation by IBMX of cGMP-binding observed with the adrenal phosphodiesterase is reminiscent of other forms of phosphodiesterases in rat platelets or lung and in rod outer segments [2–5]. They also display a cGMP-binding activity which is co-purified with a phosphodiesterase activity. However, by multiple criteria, these enzymes are different proteins. For example, only the adrenal form is sensitive to stimulation by cGMP in the phosphodiesterase reaction. Recent immunological studies have also shown that the adrenal phosphodiesterase is antigenically distinct from the rod outer segment cGMP-binding phosphodiesterase [13]. Thus so far, as shown here, the only common characteristic between these phosphodiesterase multiple forms is the IBMX stimulation of [³H]cGMP binding.

The relationship between stimulation of cGMP-binding and inhibition of phosphodiesterase activities is at present unknown. In rod outer segments, a heat-stable trypsin-labile factor is able to inhibit phosphodiesterase activity and to

stimulate the binding of cGMP to the cGMP-binding phosphodiesterase [6, 29]. Activators of the latter enzyme, i. e. light and GTP, can almost completely reverse the two reciprocal effects of the inhibitor factor [29]. It is not known whether such a factor could also control binding and hydrolytic activity associated to the adrenal cGMP-stimulated phosphodiesterase.

We have previously proposed a model for the chemical interactions between cGMP and the non-catalytic cGMP binding site [14]. These interactions were hypothetical because they were based on the assumption, validated in the present study, of a correlation between binding and phosphodiesterase activation. It is now possible to directly compare binding of analogs to activation and to assess the activation mechanism of cGMP in terms of chemical interactions.

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